PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 150, 807–809 (1975)

The Urea Requirement and Urease Production of some Human Species of T-Mycoplasmas (39129)

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(Introduced by Bernard A. Briody)

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A metabolite considered essential for the growth of the T-mycoplasmas is urea (1, 2), and in consequence it has been suggested that they be designated the ureaplasmas (3). The urea is hydrolyzed to CO_2 and NH_3 (4). T-mycoplasmas will grow in media contain-20 μg/ml urea (5). Yet the minimum concentration required for growth has not been determined. Neither has it been ascertained whether the urease is intracellular or secreted into the medium. T-mycoplasmas differ in their requirement for some unknown growth factors (6). Therefore, seven species of T-mycoplasmas have been examined for the concentration of urea required for growth and for their production of urease. The results are reported herein.

Materials and methods. The composition of the T-broth, pH 6.5, and T-agar, pH 6.5, has been reported (5, 6). Fraction A broth, pH 6.5, contained 3% trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Md.), 0.5% PPLO Serum Fraction A (Microbiological Assoc., Bethesda, Md.), and 20 μg/ml urea. The techniques for making colony counts of viable organisms (7), for preparing single-cell suspensions by sonication (8), and for obtaining growth curves are those used for the classical mycoplasmas (9) and have been described previously. The broth cultures were assayed on T-agar plates that were incubated in a 37°C humidified incubator that was gassed with 5–10% CO_2 while the broth cultures in screw-capped tubes were incubated at 37°C in a water bath.

T-mycoplasmas. The sources of the strains have been reported (5). T-mycoplasmas T-McA, T-960, and T-58 are laboratory strains, while T-210, T-213, T-220, and T-221 were isolated from patients with nonspecific urethritis.

Urease assays. A modification of Sumner's method (10) was used to ascertain the urease activity of the T-mycoplasmas. An aqueous suspension of the T-mycoplasmas was prepared and 0.2 ml was added to test tubes containing a reaction mixture consisting of 0.1 ml potassium phosphate buffer, pH 7.0, 0. 1 ml 1 *M* urea, and 0.6 ml water. The test tubes were incubated in a water bath at 25°C and, at intervals, tubes were removed, 1 ml *N* HCl added to inactivate any urease, and their NH_3 content determined by direct Nesslerization (11). The concentration of phosphate buffer and urea used in the reaction mixture, was contained in the NH_3 standards. Optical density was measured at 450 nm in a Perkin–Elmer Coleman spectrophotometer model 44. For urease assay of broth, 4 ml broth were incubated with 0.5 ml 1 *M* potassium phosphate buffer, pH 7.0, and 0.5 ml of 1 *M* urea for 4 hr at 25°C. The reactions were terminated by fast freezing in a dry ice–ethanol slurry. Included in each assay was a zero time control and a sample of uninoculated medium that had been incubated for the same period as the T-mycoplasma culture. The NH_3 content of the broth was obtained by micro Kjeldahl steam distillation into 2% boric acid containing a bromcresol green–methyl red indicator. The NH_3 content was determined by titration with previously standardized 0.01 *N* HCl. Known concentrations of NH_3 as $(NH_4)_2SO_4$ were determined before and after each series.

Results. The seven T-mycoplasma were grown routinely in Fraction A broth containing 20 μg urea/ml and reached a concentration of between 10^5 and 10^6 cells/ml broth after 24–41 hr incubation. They died in Fraction A broth without urea which indicated that urea was essential for growth and

that the other constituents of the medium did not contain either urea or a substance that could act as an alternate metabolite. When Fraction A broth containing either 10, 5, 2.5, or 1.25 μg urea/ml was inoculated with between 10^3 and 10^4 T-mycoplasmas/ml, T-960 and T-220 did not replicate significantly. The remaining strains would not grow in broth containing less than 10 μg urea/ml. Therefore an attempt was made to grow these strains in Fraction A broth containing lower concentrations of urea. Each T-mycoplasma was passaged sequentially three times in Fraction A broth containing a given concentration of urea before inoculating broth containing a lower concentration with between 1.2×10^2 and 8×10^3/ml T-mycoplasmas from the previous pass. The concentrations of urea employed were 10 μg, 5 μg, 2.5 μg, and 1.25 μg/ml of Fraction A broth. Two T-mycoplasmas, T-960 and T-220, would not grow in broth containing 10 μg per ml, while the laboratory strains T-McA and T-58, and the wild strains T-210, T-213, and T-221 were adapted to grow to over 10^5 T-mycoplasmas/ml in Fraction A broth containing 2.5 μg urea/ml but failed to grow in broth containing 1.25 μg urea/ml, indicating that the minimum amount required to reach a concentration of over 10^5/ml viable T-mycoplasmas was more than 10 μg/ml for T-960 and T-220, and over 1.25 μg for T-210, T-213, and T-221.

Urease activity. After an exponential phase Fraction A broth culture of the T-mycoplasma species had been assayed by viable counts, the culture was centrifuged at 14500*g* for 30 min to deposit the organisms, the broth decanted, and the cells resuspended in water. Their urease content was assayed by inoculating tubes of the reaction mixture and determining the amount of NH_3 produced after 20, 40, and 60 min incubation. From the viable counts it was possible to relate the production of NH_3 to the number of cells. The amount of NH_3 released by the urease in 10^4 cells has been calculated and is given in Table I.

The decanted medium was filtered through a Millipore membrane 0.22 μm pore diameter to remove any residual T-mycoplasmas and tested for sterility by inoculating T-agar plates. The urease content of the sterile broth could not be ascertained by the technique used for the T-mycoplasmas because of the precipitate formed when the Nessler's reagent and NaOH were added to the mixture. This precipitate quenched the color reaction, and this was confirmed by adding high concentrations of NH_3 to the broth mixture. Therefore, the urease content of the broth was ascertained via steam distillation. The NH_3 content was compared with that of a control of uninoculated broth. They did not differ significantly. Therefore, urease is not secreted into the medium.

TABLE I. INTRACELLULAR UREASE ACTIVITY EXPRESSED AS NANOMOLES NH_3 PER HOUR PRODUCED BY 1×10^4 T-MYCOPLASMAS

T-mycoplasma	NH_3 (nmoles)
T-McA	7.7
T-960	10.3
T-58	4.4
T-210	1.9
T-213	13.3
T-220	7.1
T-221	4.6

Discussion. T-mycoplasmas grow to the same number per ml of T-broth when the T-broth contains urea within the range 20–80 μg/ml, irrespective of the size of the inoculum (5). This indicated that even though urea is an essential metabolite, the amount of urea in the media within this range is not critical for the growth of the T-mycoplasmas. Urease rapidly hydrolyzes urea to NH_3 and CO_2. If urease was secreted into the T-broth, the amount of urea required to enable the T-mycoplasmas to reach their maximum number should vary with the number of cells in the inoculum and their ability to secrete urease. Therefore, these observations suggested that the urease remained intracellular, and this has been confirmed. However, two species of T-mycoplasmas required 20 μg urea/ml for growth while the other five species can be adapted to grow in broth containing 2.5 μg/ml broth. Therefore, T-mycoplasmas differ in their minimal requirement for urea. None of the strains tested secreted urease into the medium. Therefore, this difference

is related to the metabolism of the cell. However, the errors inherent in the laboratory techniques for counting T-mycoplasmas and obtaining cell suspensions prevents any valid correlation between their intracellular urease and requirement for urea.

Summary. Seven species of human T-mycoplasmas that grow in Fraction A and 20 μg urea/ml died when the urea was omitted. Two species would not grow in Fraction A broth containing 10 μg/urea/ml. The other five strains grew in broth containing 10 μg urea/ml and were adapted by serial passage in broth containing decreasing concentrations of urea to grow in broth containing 2.5 μg/ml urea, but not in broth containing 1.25 μg/ml. Therefore the minimal urea requirement is not the same for the growth of all strains of T-mycoplasmas. In exponential phase broth cultures, urease was detected only intracellularly, none being found in the medium.

We wish to thank Ms. Marsha Trocola for her excellent technical assistance. This research was supported by grant number RO1 A108282 from the Institute of Allergy and Infectious Diseases.

1. Ford, D. K., and MacDonald, J., *J. Bacteriol.* **93,** 1509 (1967).
2. Shepard, M. C., and Lunceford, C. D., *J. Bacteriol.* **93,** 1513 (1967).
3. Shepard, M. C., Lunceford, C. D., Ford, D. K., Purcell, R. H., Taylor-Robinson, D., Razin, S., and Black, F. T., *Int. J. System. Bacteriol.* **23,** 160 (1974).
4. Ford, D. K., McCandlish, K. L., and Gronlund, A. F., *J. Bacteriol.* **102,** 605 (1970).
5. Furness, G., *J. Infect. Dis.* **127,** 9 (1973).
6. Furness, G., *J. Infect. Dis.* **128,** 703 (1973).
7. Furness, G., Pipes, F. J., and McMurtrey, M. J., *J. Infect. Dis.* **118,** 1 (1968).
8. Furness, G., *Appl. Microbiol.* **18,** 360 (1969).
9. Furness, G., Pipes, F. J., and McMurtrey, M. J., *J. Infect. Dis.* **118,** 7 (1968).
10. Sumner, J. B., *in "Methods in Enzymology"* (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, p. 378. Academic Press, New York (1958).
11. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques," 4th ed., p. 208. Burgess, Minneapolis, (1964).

Received July 16, 1975. P.S.E.B.M. 1975, Vol. 150.